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Acrosome Reaction of Sperm in the Mud Crab *Scylla serrata* as a Sensitive Toxicity Test for Metal Exposures

Ziping Zhang · Hua Cheng · Yilei Wang · Shuhong Wang · Fangjing Xie · Shaojin Li

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Abstract In order to test the sensitivity of the sperm cell of the mud crab *Scylla serrata* to heavy metals, the toxic effects of Ag^{+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} on the acrosome reaction (AR) were studied by artificially inducing the AR of sperm exposed to heavy metals, counting the AR rates by light microscopy, and observing structural changes in sperm by transmission electron microscopy. The AR in *S. serrata* occurs at two stages. The first stage (ARI) is the eversion of the subacrosomal material. The second stage (ARII) is the ejection of the acrosomal filament. The results showed the EC_{50} values of the AR based on (ARI + ARII)% for Ag^{+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were 10.02, 2.14, 13.69, and 2.21 μg/L, and the EC_{50} values based on ARII % of Ag^{+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were 1.96, 0.20, 1.46, and 0.34 μg/L. The order of toxicity is $Cd^{2+} > Zn^{2+} > Cu^{2+} > Ag^{+}$ based on the percentage of reacted sperm at the second stage. Sperm cells exposed to heavy metals showed an increased rate of swelling, shape irregularities, and the acrosomal filament of some sperm cells was, crooked, ruptured, and even dissolved. The AR of the sperm cell from *S. serrata* is more sensitive to the tested heavy metals compared to sea urchin sperm cell toxicity tests.

Z. Zhang · H. Cheng · Y. Wang (✉) · S. Wang · F. Xie
The Key Laboratory of Science and Technology for Aquaculture and Food Safety, Fisheries College, Jimei University, 361021 Xiamen, China
e-mail: ylwang@jmu.edu.cn

Present Address:
Z. Zhang
Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX 78666, USA

Present Address:
H. Cheng
Department of Biostatistics, School of Public Health, Louisiana State University, New Orleans, LA 70112, USA

S. Li
Oceanography Department, Xiamen University, 361005 Xiamen, China

The mud crab *Scylla serrata* is an economically important crab in southern China. To date, the larvae supplies for crab aquaculture are mainly dependent on natural stocks because artificial propagation is still at the laboratory stage. Unfortunately, the habitat of *S. serrata* is threatened by many pollutants including heavy metals (Phavale 1990). Weng and Li (1996) have studied the effects of heavy metals on the larva of *S. serrata*, but there are no studies that explore whether the sperm of *S. serrata* are vulnerable to heavy metal exposure.

The importance of acrosome reaction (AR) in fertilization success has been investigated and reviewed intensively and extensively (Fickel et al. 2007; Wassarman 1999). In recent decades, much research has been devoted to reveal that sperms of the decapods need to undergo AR before penetrating the eggs for fertilization success (Griffin and Clark 1990; Leung-Trujillo and Lawrence 1987). Thus, evaluation of the fertilization capability based on the induction of the AR have been successfully set up and used in mammalian system (Clarke et al. 2006; do Nascimento et al. 2007) as well as other models such as crab (Bhavanishankar and Subramoniam 1997), shrimp (Diwan and Joseph 2000), and sea urchin (Schuel et al. 1994). Pillai et al. (1997) used the AR as a direct measurement of the impact of lignin-derived macromolecules on sea urchin sperm. They demonstrated that the AR of the sea urchin

can be used as a model to investigate the action of toxic substances at the subcellular level. The sperm of *S. serrata* have no flagella and belongs to typical nonmobile sperm. The viability of the sperm can be assessed by following the AR sequence under a microscope (Bhavanishankar and Subramoniam 1997; Wang et al. 2001). The AR of *S. serrata* is a biphasic event that generates forward movement for the nonmobile sperm to penetrate the chorion of the egg, so it is a crucial event for successful fertilization (Wang et al. 2001). Reduction of sperm function will decrease fertilization efficiency; as a result, it will reduce not only recruitment for aquaculture but also population replenishment in a natural environment. AR is potentially a good indicator of impaired function in the sperm of *S. serrata* and, hence, might be useful as a bioindicator to monitor heavy metal contamination in natural environments. In this study, the AR is utilized in vitro as an experimental cellular model to investigate the toxic effects of heavy metals on the sperm cell of *S. serrata*. It will provide toxicity information for the resource protection of *S. serrata* and other marine organisms.

From the 1980s, most investigations about sea urchin gametes concentrated on ecotoxicological testing and monitoring. The sea urchin sperm cell became a useful tool for ecotoxicologists, and many standard test protocols were written in the 1980s, 1990s, and 2000s (Chapman 1995; Dinnel 1995; Ghirardini et al. 2005). However, all species of sea urchin live only in the marine environment; thus, the sperm cell tests can only be used for seawater. Fortunately, crabs with a worldwide distribution can live in either seawater or freshwater. If the sperm cell test presented here can be established and standardized, it will be extended not only to other marine crab species but also to freshwater crab species; thus, a new bioassay will be developed to monitor not only saltwater but also freshwater environmental pollution.

Materials and Methods

Animals

It was well documented that processes of maturation or capacitation in thelycum of female animal are required for the sperms of decapods to reach fertilization competence (Alfaro et al. 2003, 2007; Clark and Griffin 1988; Griffin and Clark 1990; Pongtippatee et al. 2007; Shigekawa and Clark 1986). Our preliminary results have also demonstrated that the AR rate of sperms directly collected from male mud crabs was very low (Wang et al. 2001). In this project, mature females of *S. serrata* were purchased from a local market of Jimei, Xiamen during the breeding season for our experiments.

Experimental Solutions

Calcium-free artificial seawater (CFASW; pH 7.2) was prepared according to the work of Clark et al. (1984). It was used for sperm collection and storage because calcium ions in natural sea water might induce AR (Wang et al. 2001). The pH of the CFASW was adjusted to 9.0 by adding freshly prepared saturated Tris–HCl solution for AR according to our previous work (Wang et al. 2001). The temperature of CFASW was 22°C ± 2°C.

In *S. serrata*, it is difficult to acquire mature eggs for artificial fertilization because females migrate to the sea for spawning (Hill 1994). To avoid this technical difficulty, we used the divalent cation ionophore A23187 to induce AR in vitro (Lindsay and Clark 1992; Talbot et al. 1976; Wang et al. 2001). The stock solution of divalent cation ionophore A23187 (Sigma) was dissolved in dimethylsulfoxide (DMSO; Sigma) at a concentration of 2 mg/mL and stored at −20°C. A23187 was diluted to working concentration of 640 μg/mL with CFASW (pH 9.0) prior to the treatment of sperm cells.

All heavy metal and Ca^{2+} stock solutions were prepared in double-distilled deionized water. The heavy metal working solutions were serially diluted with CFASW (pH 9.0) to achieve a 2× exposure concentration of 1.12, 2, 3.4, 6.4, 11.2, 20, and 36 μg/L for Ag^{+}, 0.36, 0.64, 1.12, 2, 3.68, 6.42, and 11.2 μg/L for Cd^{2+} and Zn^{2+}, and 2, 3.6, 6.4, 11.2, 20, 36, and 64 μg/L for Cu^{2+}. These working solutions will be mixed with equal volumes of sperm suspension solution to form a final concentration of 1× exposure. All of the containers used in the experiments were prefilled with the corresponding experimental solution overnight to saturate the adsorption sites of the container walls. Before the real exposure, these containers were rinsed with the corresponding experimental solution again to minimize the differences between nominal and actual metal concentration in experiments. Exposure heavy metal concentrations are nominal, not measured. The final working concentration of Ca^{2+} was 2.5 g/100 mL.

Sperm Collection

Spermathecae in female *S. serrata* were cut open and spermatophores were extracted. Filter paper was used to remove water on the surface of the spermatophors. Immediately after water removal, the spermatophores were gently homogenized in a glass tissue grinder with 50–100 μL of CFASW (pH 7.2) until all of the visible tissue had been completely homogenized. CFASW containing sperm were carefully transferred to a 0.5-mL centrifuge tube. The sperm suspension was then centrifuged at 500*g* for 3–5 min to pellet the sperm. The supernatant was removed and the sperm pellet was resuspended with

CFASW. The density of the sperm cell suspension was determined by a hemacytometer; it was then adjusted to form a suspension with a concentration of 1.6×10^8 sperm cell/mL and stored at 4°C for further experiments. It took 60 min to complete the sperm cell suspension preparation. We used freshly prepared (less then 5 h after preparation) sperm suspension solution for the experiments. Spermatophores from five females of *S. serrata* were mixed and used for each experiment.

Dose–Response Study

In our previous work (Wang et al. 2001), three factors with five levels including a series of pH (6.0, 7.0, 8.0, 9.0, and 10.0), a series of different concentrations in A23187 (32, 40, 48, 56, and 64 μg/mL), and Ca^{2+} (0.10, 0.15, 0.20, 0.25, and 0.30 g/100 mL) were chosen to assay the optimal condition for the AR of sperm with an orthogonal experiment. The results showed that the highest rate of the AR of sperm can be achieved when sperms isolated from the spermathecae of the female crab were exposed to 64 μg/mL A23187, 0.25 g/100 mL Ca^{2+} at CFASW (pH 9) for 40 min. According to the optimal condition, experiments in the present study were carried out in 1.5-mL microcentrifuge tubes. The total reaction volume of 500 μL containing 250 μL of 2× heavy metal working solutions, 50 μL of 2.5 g/100 mL Ca^{2+}, 50 μL of 640 μg/mL A23187, 10 μL sperm suspension, and 140 μL of CFASW (pH 9.0), in which final serial heavy metal concentrations were 0.56, 1, 1.8, 3.2, 5.6, 10, and 18 μg/L for Ag^{+}, 0.18, 0.32, 0.56, 1, 1.8, and 3.2 5.6 μg/L for Cd^{2+} and Zn^{2+}, and 1, 1.8, 3.2, 5.6, 10, 18, and 32 μg/L for Cu^{2+}. For the control test, 250 μL of 2× heavy metal working solution was replaced with CFASW (pH 9.0). After the reaction, the sperm were fixed by adding an equal volume of 6% glutaraldehyde (buffered with CFASW). All fixed samples were stored in the refrigerator (4°C) for AR counting or postfixation. All experiments were repeated six times, with three replicates per treatment in each experiment.

The AR was observed and counted under a light microscope at a magnification range of 1000 × 2,500. An aliquot of sperm suspension was taken from each test tube and 200 sperm cells were counted randomly. The AR in *S. serrata* occurs at two stages. According to Wang et al. (2001), the most important character of the first stage is the eversion of the subacrosomal material, and the salient change in the second stage is the ejection of the acrosomal filament (Fig. 1). Reacted sperm with everted vesicles were counted as being in the first stage of the AR (ARI); the sperm completing total AR were counted as sperm in the second stage of AR (ARII). The total acrosome-reacted sperm were presented as the sum of sperm at the ARI and ARII stages.

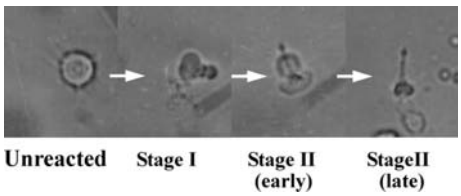

Fig. 1 The AR in the sperm of mud crab *S. serrata* proceeds on two stages; stage I is the eversion of the subacrosomal material and stage II is the ejection of the acrosomal filament. Magnification: 2,000×

Ultrastructure Study

After the reaction, the sperm of both the control and experimental samples exposed near the median effect concentrations (EC_{50}) were treated by the following procedure for ultrastructure examination. The chosen concentrations of Ag^{+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were 1.8, 0.18, 1, and 0.32 μg/L, respectively. The samples were fixed by adding an equal volume of 6% glutaraldehyde (buffered with CFASW, pH 7.2) for 2 h. Fixed samples were postfixed in 1.5% aqueous OSO_4 for 2 h, then dehydrated in acetone, and embedded in an epoxy resin. The embedded samples were cut with glass knives (KLB-8800). Ultrathin sections were stained with uranyl acetate and lead citrate and observed under transmission electron microscope (JEM-CXII).

Data Analyses

Means and standard deviations were computed for each test response. One-way analysis of variance (ANOVA) was used to test the null hypothesis that treatments do not cause a significant change in the percentage of sperm in the first stage. Regression analysis was utilized to examine the response of the different types of ARs to variable metal concentrations and to calculate the EC_{50} values.

Results

Heavy metals significantly reduced the AR compared with the control group. Both the percentage of acrosome-reacted sperm ([ARI + ARII]%) and the percentage of sperm at the second stage (ARII %) decreased as the concentration of heavy metals increased, but the percentage of sperm at the first stage (ARI%) did not significantly change (Fig. 2). Regression analysis clearly showed that (ARI + ARII)% and ARII % exhibited a good dose response to the concentration of heavy metals ($p < 0.002$) (Table 1).

The EC_{50} values of heavy metals for the AR in the sperm of *S. serrata* were calculated and are listed in Table 2. The percentage of ARI + ARII changed

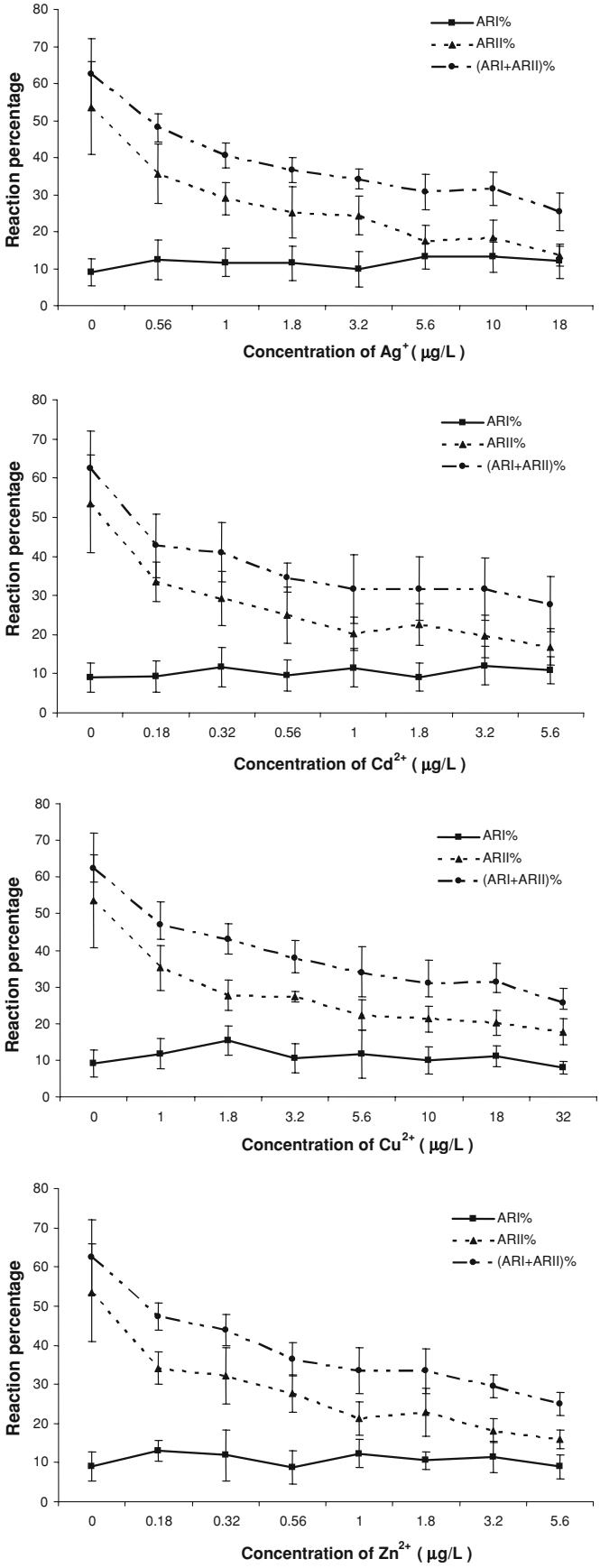

Fig. 2 The effects of heavy metals on the AR in *S. serrata*. Each value is a mean of six experiments. The unit of concentration is micrograms per liter. Error bar = SD. All means are significantly different compared to control at $p < 0.02$. ARI % stands for the percentage of sperms in first stage, ARII% stands for the percentage of sperms in the second stage, and (ARI + ARII)% stands for the percentage of sperms in stage I and stage II together. $n = 6$

Table 1 Dose–response correlation of toxicity of heavy metals to the RA of sperms in *S. serrata*

Heavy metals	Regression equations		R^2	p
Ag^{+}	ARII%	$Y = 30.2 - 13.6X$	0.947	0.000
	(ARI + ARII)%	$Y = 41.9 - 13.2X$	0.927	0.001
Cd^{2+}	ARII%	$Y = 23.9 - 10.2X$	0.891	0.001
	(ARI + ARII)%	$Y = 34.4 - 9.53X$	0.877	0.002
Cu^{2+}	ARII%	$Y = 32.4 - 10.5X$	0.905	0.001
	(ARI + ARII)%	$Y = 45.8 - 13.4X$	0.956	0.000
Zn^{2+}	ARII%	$Y = 24.6 - 12.5X$	0.944	0.000
	(ARI + ARII)%	$Y = 35.6 - 14.1X$	0.949	0.000

Note: X is the logarithm of the concentration of heavy metal; Y is the relative AR%. The first stage (ARI) is the eversion of the subacrosomal material and the second stage (ARII) is the ejection of the acrosomal filament. $n = 6$

significantly even when concentrations of heavy metals were less than 1 μg/L. The EC_{50} based on ARII % were as follows: Ag^{+}, 1.96 μg/L; Cd^{2+}, 0.20 μg/L; Cu^{2+}, 1.46 μg/L; and Zn^{2+}, 0.34 μg/L. The results based on (ARI + ARII)% for Ag^{+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were 10.02, 2.14, 13.69, and 2.21 μg/L, respectively. The reaction percentage of sperm in the second stage is a more sensitive than that of the total acrosome-reacted sperm.

According to the EC_{50} values, the order of toxicity was $Cd^{2+} > Zn^{2+} > Cu^{2+} > Ag^{+}$ based on the percentage of reacted sperm at the second stage, whereas the order of toxicity was $Cd^{2+} > Zn^{2+} > Ag^{+} > Cu^{2+}$ based on the percentage of all acrosome-reacted sperm.

The observation of sperm ultrastructure by transmission electron microscope showed that sperm acrosome membrane was intact and the acrosomal filament of perforatorium was straight and arranged regularly with high electron density in the control sample (Fig. 3a).Compared to control sperm, adverse effects were evident in all experimental samples: Sperm cells became swollen, irregular, and less electron dense. The damages were prominent in the acrosomal regions; the acrosomal filament of some sperm cells was crooked, ruptured, and even dissolved (Fig. 3b–e). There were no significant differences in spermatozoa exposed to all heavy metals.

Discussion

Effects of Heavy Metals on Different Stages of the Acrosome Reaction

When counting all sperm cells in the reaction, the percentage of the spermatozoa at the second stage decreased with the increasing concentration of heavy metals, but the percentage of sperm at the first stage did not change significantly (Fig. 2). If only the reacted sperms [(ARI + ARII)%] are considered, the percentage of the sperm staying in the first stage [(ARI/(ARI + ARII)] increased. The reaction percentage of AR and the concentration of heavy metal showed a strong linear correlation (Table 1). The increased ratio of sperm cells that stagnated in the first stage suggests that heavy metals might play a critical role in inhibiting the process of AR, especially the processing course from the first stage to the second stage. Ultrastructural observations suggested that heavy metals likely restrict the extension of acrosomal filament by destroying the structure of canaliculus or affecting the constriction component in the acrosomal filament, as both of these components participate in

Table 2 Comparative EC_{50} μg /L values (mean ± standard deviation or 95% confidence interval) of heavy metals of sperm cell test with *S. serrata* and some echinoid species

Species	Ag^{+}	Cd^{2+}	Cu^{2+}	Zn^{2+}	Investigators
S. serrata (ARII)	1.96 (1.56–2.47)	0.20 (0.13–0.29)	1.46 (0.98–2.16)	0.34 (0.25–0.47)	Zhang et al. (this article)
S. serrata (ARI + ARII)	10.02 (7.96–12.63)	2.14 (1.40–3.28)	13.69 (10.05–18.65)	2.21 (1.67–2.92)	Zhang et al. (this article)
Strongylocentrotus purpuratus	115 (102–131)	18,000 (15,000–23,000)	25 (12–60)	262 (221–312)	Dinnel et al. (1989)
S. droebachiensis	86 (71–105)	26,000 (21,000–32,000)	59 (51–68)	383 (302–491)	Dinnel et al. 1989
S. franciscanus	112 (N.C.)	12,000 (8,000–20,000)	1.9 (1.6–2.5)	313 (261–378)	Dinnel et al. (1989)
Arbacia spatuligera	NT	140,900 ± 88,100	18 ± 10	116 ± 61	Larrain et al. (1999)
A. punctulata	NT	38,000 (34,300–42,500)	12 (11 ± 12)	121 (110 ± 113)	Burgess et al. (1993)
Dendraster excentricus	54 (42–70)	8,000 (4,000–15,000)	26 (18–37)	28 (10–76)	Dinnel et al. (1989)
Paracentrotus lividus	NT	8,400 (7,250–9,740)	57 (45–69)	210 (200–230)	Novelli et al. (2003)
Peronella japonica	NT	NT	5	5	Kobayashi (1980)
Heliocidaris erythrogramma	NT	NT	1	NT	Kobayashi (1980)
Anthocidaris crassispina	NT	1,700	NT	NT	Vaschenko et al. (1999)
Echinometra mathaei	NT	>100	14	NT	Ringwood (1992)

Note: *N.C.* not calculated, *NT* not tested

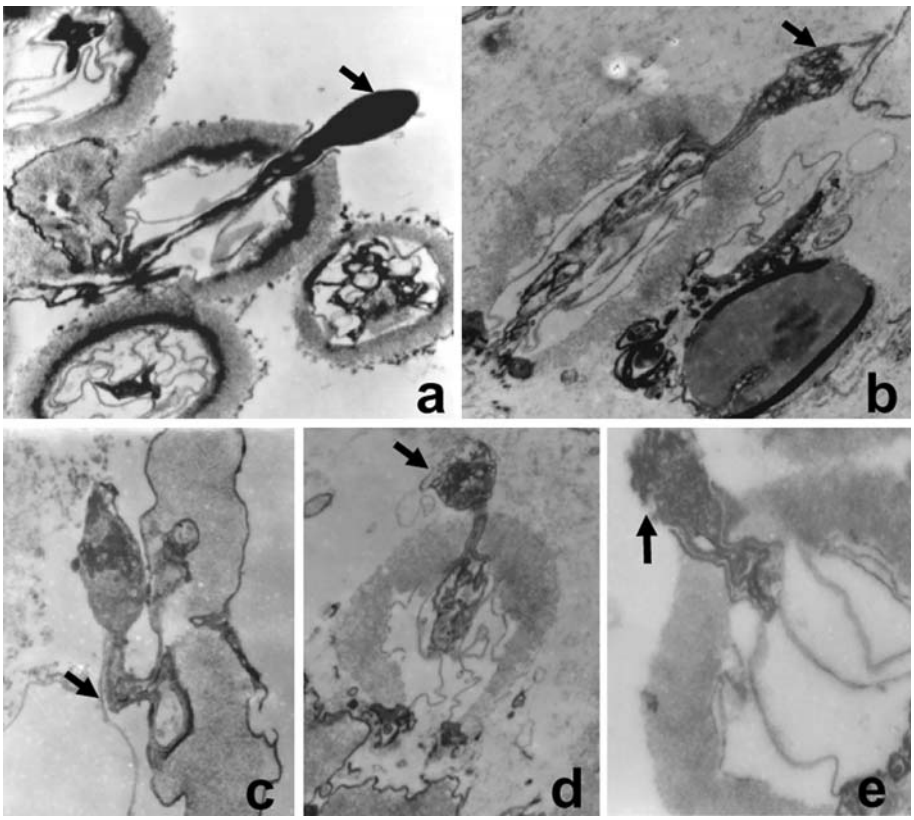

Fig. 3 Longitudinal section of sperm in the second stage from control group sample and exposure group sample. **a** The sperm in the second stage from control group sample; the arrow shows that the structure of acrosomal filament was intact and highly electron dense. Magnification: 10,000×. **b–e** The sperm in the second stage from Ag^{+} (1.8 μg /L), Zn^{2+} (0.32 μg/L), Cd^{2+} (0.18 μg /L), and Cu^{2+} (1 μg /L) exposure group sample, respectively; the *arrows* show that the acrosomal filament was destroyed (less electron dense, crooked, dissolved, and broken). Magnification: 10,000×

extending the process of AR. The acrosomal filament of sperm cells exposed to heavy metals became less electron dense, which is indicative of the destruction of the acrosomal filament (Fig. 3b–3e).

We also found that the toxicity of the studied heavy metals in descending order with respect to the total reaction percentage of sperm cells was $Cd^{2+} > Zn^{2+} > Ag^{+} > Cu^{2+}$, whereas the order with respect to the percentage of the sperm cells in the second stage was $Cd^{2+} > Zn^{2+} > Cu^{2+} > Ag^{+}$. This difference indicates that Cu^{2+} inhibits the process from the first stage to the second stage more effectively than Ag^{+} does.

Spermatozoa Is More Sensitive to Heavy Metals Compared to Larva at Different Developmental Stages

The sea urchin is widely used in metals toxicity research. Kobayashi (1980) compared the effects of Cu^{2+} on various early developmental stages of two echinoids species (*Peronella japonica* and *Heliocidaris erythrogramma*). Based on the minimum effect concentration, sperm was most sensitive in *P. japonica*, whereas in *H. erythrogramma*, metamorphosis was the most sensitive stage. In *Arbacia lixula*, the effect of Zn^{2+} on development is that the percentage of motile sperm decreases significantly when compared to a control group, but there are no effects on adults even after being exposed to 1 mg/L Zn^{2+} for 20 days (Castagna et al. 1981). Dinnel et al. (1989) showed that sea urchin sperm toxicity bioassay is not more sensitive to heavy metals than the sea urchin embryo bioassay. Similarly, in *Paracentrotus lividus*, the embryo was more sensitive to Cd^{2+} and Zn^{2+} than the sperm cell, but both the embryo and spermatozoa were similarly sensitive to Cu^{2+} (Novelli et al. 2003). However, after reviewing more than 20 years of experimental data, Dinnel (1995) concluded that sperm tests are sensitive to many toxicants, generally more sensitive to complex effluents than adult animal tests, and almost as sensitive as marine embryo and larval development tests requiring 2–4 days of exposure. In fish, *Antherinops affinis*, the sensitivity order was sperm > embryo > juveniles, which is supported by evidence from another group (Anderson et al. 1991).

Weng and Li (1996) studied the effect of several heavy metals on the juveniles of *S. serrata* and reported that zoea III stage (Z_3) and zoea I stage (Z_1) were the two most sensitive stages. However, the LC_{50}'s of Cd^{2+}, Cu^{2+}, and Zn^{2+} for Z_1 during 24-h experiments was 2.217 μM (~249 μg/L), 0.8530 μM (~54 μg/L), and 37.30 μM (~2,439 μg/L); for Z_3, they were 1.095 μM (~123 μg/L), 0.6430 μM (~40 μg/L), and 18.96 μM (~1,240 μg/L),

respectively. All values (Weng and Li 1996) were higher than the results from this study, which reveal that the sperm is more sensitive to heavy metals than larva. Our results are comparable with the results on sea urchins.

A probable reason for the sensitivity of sperm is that they lack a detoxification system. For example, no lysosome has been found in spermatozoa of *S. serrata* (Wang et al. 1997), but the larva might have a detoxification system similar to other crabs (Sanders et al. 1983). Metal-binding proteins, especially metallothionein (MT), have been studied and their detoxification functions have been confirmed in larvae of some organisms. For example, Damiens et al. (2006) reported that MT induction in larva of bivalve mollusks was strongly correlated with increasing metal contents. However, to date, similar metal-binding proteins or MT have not been reported in the sperm of crustaceans.

Comparison Between Sperms of *S. serrata* and Sea Urchins

Most of the studies on metal toxicity to sperm have been conducted on sea urchins. In sea urchins, the range of EC_{50}'s of Ag^{+}, Cd^{2+}, Zn^{2+}, and Cu^{2+} were 54–115, 100–140, 900, 28–383, and 1–59 μg/L, respectively (Burgess et al. 1993; Dinnel et al. 1989; Kobayashi 1980; Larrain et al. 1999; Novelli et al. 2003; Ringwood 1992; Vaschenko et al. 1999). The EC_{50} (ARII)% of Ag^{+}, Cd^{2+}, Cu^{2+}, Zn^{2+} in the AR of *S. serrata* were 1.96, 0.2, 1.46, and 0.34 μg/L respectively. These values are all distinctly lower than those reported for sea urchins (Table 2).

The experimental system in *S. serrata* sperm is different from that of the sea urchin. In the sea urchin sperm fertilization bioassay, sperm cells are exposed to test waters or toxicants for a fixed period and then used to fertilize unexposed eggs, with fertilization efficiency assessed by counting the proportion of eggs with a raised fertilization membrane (Dinnel 1995; Vaschenko et al. 1999). In the present study, the sperms of *S. serrata* were exposed to heavy metals directly and then the AR rates were assessed.

In addition, sperm motility is an observed end point in current sperm cell toxicity tests (Dinnel et al. 1989). Pillai et al. (1997) found that lignin-derived macromolecules (LDMs) inhibited the AR in sea urchins without affecting sperm motility, which indicated that the LDMs had harmed the sperms before the motility ability change could be detected. This indicates that the AR is more sensitive than sperm motility. If heavy metals in the AR share the same mechanism of LDMs (i.e., metals affect the AR before they affect motility), then it is reasonable to suspect that the sperms of *S. serrata* show higher sensitivity than sperms of echinoids by comparing their EC_{50} values.

Furthermore, the sperm–egg ratio is a well known difficulty of the sea urchin sperm fertilization bioassay (Lera et al. 2006; Vazquez 2003). The present experimental system uses only one gamete and does not require fertilization to assess the effects of heavy metals. The method designed in this study is simpler than the current sperm/fertilization method in the sea urchin.

Taken together, our results demonstrate that the AR of sperm from *S. serrata* is more sensitive to heavy metals than the sea urchin sperm bioassay, implying that the AR of *S. serrata* could be developed to be a useful bioassay of heavy metal exposure for further fertilization study.

Mechanisms of Heavy Metal Effects on Acrosome Reaction

Although it is not the focus at present to specify the particular sites within the spermatozoa of *S. serrata* on which these metal ions exert their action, results in this article show some insight into the mechanism of heavy metals effects on the AR of *S. serrata*.

To date, there is no clear understanding of the toxicity mechanism of heavy metals on sperm. Two major models have been proposed for the uptake of heavy metals by aquatic organisms. One is a facilitated diffusion model, which does not necessarily require energy (Rainbow and Dallinger 1993; Simkiss and Taylor 1989). The other model involves energy-requiring ionic pumps, such as the Na^{+}/K^{+} ATPase system; heavy metals can be incorporated into such pumps (Simkiss and Taylor 1989). By the facilitated diffusion model, internal metal concentrations can be orders of magnitude higher than externally, and heavy metals might still be taken up passively. This is consistent with both the dose–response relationship and the structural changes (Fig. 3b–e) detected in the present study. The treated sperm cells were swollen and less electron dense and some of the acrosomal filaments were even broken (Fig. 3b–e). This corresponds to the sea urchin sperm exposed to Cd^{2+} that were also discovered to be less electron dense and more swollen (Au et al. 2000).

Heavy metals, such as Zn^{2+} or Cu^{2+}, can interact with soluble sulfhydryl groups of protein to form mercaptides to block the active sites on contractile proteins of spermatozoa then to inhibit the motility of spermatozoa (Young and Nelson 1974). As nonmotile decapods spermatozoa, the number of mitochondria in the spermatozoa of *S. serrata* is significantly lower than motile spermatozoa (Wang et al. 1997). However, acrosomal filaments, which are essential for AR, are very similar to contractile proteins in structure. The active sites of the acrosomal filaments could be blocked by heavy metals in a similar way, and led to the proceeding of AR were inhibited.

Another possible mechanism of inhibition of AR by heavy metals might relate to Ca^{2+} flow. The most important change during the AR process is that the Ca^{2+} channel in the membrane of the sperm cell opens, Ca^{2+} flows inside, and the intracellular concentration of Ca^{2+} increases. This is a requisite of the AR. Heavy metals might inhibit the Ca^{2+} influx and the Ca^{2+} channels involved in the influx (Darszon et al. 1999; Liévano et al. 1990; González-Martínez et al. 2001). Rogers and Yanagimachi (1976) reported the competitive effect between magnesium and calcium in the AR in guinea pigs and speculated that magnesium competed with calcium for carriers on the plasma membrane. Liévano et al. (1990) confirmed this result in sea urchin sperm. In addition, Liévano et al. (1990) detected that both Cd^{2+} and Co^{2+} blocked Ca^{2+} channels and inhibited the AR. Based on the above information and the results of the present study, it is reasonable to generate a hypothesis that the competition on the Ca^{2+} channel between Ca^{2+} and heavy metals inhibits the influx of Ca^{2+} and that the concentration of Ca^{2+} in the sperm cannot reach the required level, resulting in AR inhibition.

Conclusion

In the present study, we describe a cellular toxicity assessment system using the AR of *S. serrata.* This is the first time that the effects of heavy metals on the sperm of a crustacean have been investigated. The AR of *S. serrata* sperm is a critical event of fertilization before the combination of sperm and egg, which includes two stages and can be easily observed under the microscope. The AR of the sperm of *S. serrata* showed high sensitivity to heavy metals and all of the EC_{50} values from present study are around the Sea Water Quality Standard (GB 3097-1997) of China for Heavy Metals (Zhao and Kong 2000). One goal of environmental monitoring is to detect contaminants before they are present at harmful effects. The high sensitivity of *S. serrata* sperm to heavy metals can justify this monitoring criterion. The experimental system designed in this study has several advantages: small water sample, short test time, simple operation, and easily detection. The bioassay will be very useful for monitoring for the presence of heavy metal contaminants in specific areas or concentration changes from one area to another, as the experiment requires 40 min.

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